

REMARKS

I. CLAIM STATUS

Claims 1-30 were pending in this application when last examined.

Claim 1-5, 7, 9, 10, 23, 24 and 26-28 are rejected.

Claims 8, 11-22, 25, 29 and 30 have been withdrawn as non-elected subject matter.

Claims 6 is objected to.

New claims 31-33 have been added by this amendment.

Upon entry of the present amendment, claims 1-33 will be pending in this application.

Claims 2, 4, and 5 have been amended.

Claim 2 was amended to recite "which has a dissociation constant of" instead of "which shows a dissociation constant of" to utilize more conventional language. Similarly, claims 4 and 5 were amended to recite "having a molecular weight" instead of "showing a molecular weight" to utilize more conventional language. Support for these editorial changes can be found in the original claims, and in the specification, for example, at page 13, lines 14-16 and at page 14, lines 6-11.

Support for new claims 31, 32, and 33 can be found in the specification, for example, at page 44, lines 10-23, at page 10, line 19 to page 11, line 18, and at page 23, line 16 to page 26, line 10, respectively.

Therefore, no new matter has been added by this amendment.

II. REJECTION UNDER 35 U.S.C. § 102

Claims 1-5, 7, 9 and 10 remain rejected under 35 U.S.C. § 102(b) as anticipated by Shimomura, EP 0 596 524 as evidenced by Goldsby, IMMUNOLOGY, 5th ed., pp. 137-139 (2000). See pages 2-3 of the Office Action.

Applicants respectfully traverse this rejection for the same reasons set forth on page 10 of the response filed March 19, 2004 and for following reasons.

To anticipate a claim, a cited prior art reference must either expressly or inherently teach each and every element of the claimed invention.

In this case, the claims call for an antibody that recognizes active HGFA and does not substantially recognize inactive HGFA. Shimomura EP '524 fails disclose an antibody that does not substantially recognize inactive HGFA as claimed.

In the response filed March 19, 2004, it was argued that Shimomura EP '524 neither expressly nor inherently teaches an antibody that does not substantially recognize inactive HGFA as evidenced by the Miyazawa reference (J. BIOL. CHEM., vol. 271, no. 7, pp. 3615-3618 (1996)). Miyazawa was cited as demonstrating that known HGFA antibodies, including the Shimomura EP '524 antibody, react with both active HGFA and inactive HGFA. In contrast, the antibodies of the claimed invention do not substantially recognize inactive HGFA. The Examiner was unpersuaded by this argument, because it was unclear that the antibodies described in Miyazawa include the Shimomura EP '524 antibody. In this regard, the Examiner requested data directly comparing the claimed antibody with the antibody disclosed in Shimomura EP '524.

Attached herewith is a Declaration under 37 C.F.R. § 1.132 by Mr. Shimomura, a co-inventor of Shimomura EP '524 and a co-author of Miyazawa reference. The Shimomura Declaration confirms that the antibodies against HGFA described in Shimomura (EP '524) are the same as those described in the Miyazawa reference (i.e., 7E10, P1-4, A-1, A-6, A-23, A-32, A-51, A-75) known in the art to recognize HGFA.

The Shimomura Declaration confirms that the analysis performed in the Miyazawa reference and in Example 4 of the present application at page 52, line 6 to page 53, line 20, demonstrates that antibody of Shimomura EP '524 recognizes both active and inactive HGFA in contrast with the antibody of the claimed invention. In view of this Declaration, it is evident that the Miyazawa reference includes the antibody of Shimomura EP '524, and this antibody reacts with both active HGFA and inactive HGFA.

Further enclosed herewith is Declaration under 37 C.F.R. § 1.132 by Mr. Naka, a co-inventor the present application. The Naka Declaration discusses a study describing the preparation of the claimed antibodies with the monoclonal antibodies described in Miyazawa and Shimomura EP '524 (i.e., 7E10, P1-4, A-1, A-6, A-23, A-32, A-51, A-75) as known in the art to recognize HGFA. This

Declaration analyzes the reaction specificity for the antibodies described in Miyazawa and Shimomura EP '524 and compares them with those of the claimed invention.

The Naka Declaration confirms that the methods for preparing the prior art monoclonal antibodies differs from that of the claimed invention. See item 1 on pages 2-3 of the Naka Declaration. The method of the claimed invention screened for and selected only three hybridomas that produce monoclonal antibodies that recognize active HGFA and do not recognize inactive HGFA from about 12,000 clones. The prior art antibodies were prepared by standard techniques, and not screened. As such, the monoclonal antibodies of the prior art recognize both active HGFA and inactive HGFA. Thus, it is clear that the antibody of the present invention could not be obtained without performing screening with a definite and sure object to obtain an antibody which specifically recognizes the active HGFA.

More importantly the Naka Declaration confirms that the prior art antibodies recognize with **both** active **and** inactive HGFA, whereas the antibodies of the claimed invention do not substantially recognize inactive HGFA. See item 2 on pages 3-4 of the Naka Declaration. Specifically, ELISA studies confirm that the monoclonal antibodies derived from the deposited hybridoma clone AHGA-A, Deposit Accession No. FERM BP-7779, were reactive for active HGFA and not inactive HGFA. The prior art monoclonal antibodies 7E10, P1-4, A-1, A-6, A-23, A-32, A-51, A-75 were reactive with **both** active HGFA **and** inactive HGFA.

Based on the above, it is clear that Shimomura EP '524 discloses antibodies that are fundamentally different and distinct from those in the claimed invention.

Furthermore, Shimomura EP '524 does not disclose an antibody with dissociation constant of 1×10^{-9} M or lower.

Accordingly, Shimomura EP '524 fails to teach or suggest an antibody that recognizes active HGFA and does not substantially recognize inactive HGFA.

Therefore, in view of the above, the rejection of claims 1-5, 7, 9 and 10 under 35 U.S.C. § 102(b) is untenable and should be withdrawn.

III. REJECTION UNDER 35 U.S.C. § 103

Claims 23, 24 and 26-28 remain rejected under 35 U.S.C. § 103(a) as obvious over Shimomura EP '524 in view of Zuk, U.S. Patent No. 4,281,061. See item 6 on page 4.

This rejection is respectfully traversed for the same reason set forth immediately above and for the reasons discussed below.

To establish obviousness, three criteria must be met. First, the prior art references must teach or suggest each and every element of the claimed invention. Second, there must be some suggestion or motivation in the references to either modify or combine the reference teachings to arrive at the claimed invention. Third, the prior art must provide a reasonable expectation of success.

In this case, the rejection relies solely upon the primary reference of Shimomura EP '524 for disclosing antibodies that recognize HGFA. However, as discussed above, Shimomura EP '524 discloses antibodies that recognize **both** active **and** inactive HGFA. Thus, Shimomura EP '524 fails to teach and/or suggest an antibodies that do not substantially recognize inactive HGFA as claimed.

Zuk fails to remedy this deficiency, because this reference is silent as to antibodies reactive to HGFA.

Thus, in view of the arguments above and the attached Declarations, the cited references fail to teach and/or suggest each and every element of the claimed invention.

Therefore, the rejection of claims 23, 24 and 26-28 under 35 U.S.C. § 103(a) is untenable and should be withdrawn.

IV. OBJECTION TO THE CLAIM

Claim 6 is objected to as dependent upon a rejected base claim 1, but would be allowable if rewritten in independent form including all the limitations of the base claim. See item 8 on page 5. In view of the argument set forth above obviating the rejection of claim 1, the objection to claim 6 is no longer tenable and should be withdrawn.

CONCLUSION

In view of the foregoing remarks, Applicants submit that the present application is in condition for allowance and notice to that effect is hereby requested.

If it is determined that the application is not in condition for allowance, the Examiner is invited to telephone the undersigned attorney at the number below to expedite prosecution of the present application.

Respectfully submitted,

Daiji NAKA et al.

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THE COMMISSIONER IS AUTHORIZED
TO CHARGE ANY DEFICIENCY IN THE
FEES FOR THIS PAPER TO DEPOSIT
ACCOUNT NO. 23-0975

ATTACHMENTS

1. Declaration Under 37 C.F.R. § 1.132, by Mr. Takeshi Shimomura (5 pp.)
2. Declaration Under 37 C.F.R. § 1.132, by Mr. Daiji Naka (6 pp.)



IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant : Naka, et al.
Appl. No. : 10/000,096
Filed : December 4, 2001
For : SPECIFIC ANTIBODY DIRECTED TO ACTIVE HEPATOCYTE
GROWTH FACTOR ACTIVATOR AND METHOD FOR USING THE
SAME

DECLARATION UNDER 37 C.F.R. § 1.132

Assistant Commissioner for Patents
Alexandria, VA 22313-1450

Dear Sir:

I, Takeshi Shimomura, declare as follows.

1. I am a graduate of Kyushu University, and where I received my doctorate degree.
2. I have been employed by Mitsubishi Kasei Corporation (currently Mitsubishi Chemical Corporation), Yokohama-shi, Kanagawa, Japan, and Mitsubishi-Tokyo Pharmaceuticals, Inc. (currently Mitsubishi Pharma Corporation), Chuo-Ku, Osaka, Japan as a researcher in the field of pharmaceutical development using cell culture techniques and gene manipulation techniques.
3. I am a co-author of the document "J. Biol. Chem., 271:3615-3618 (1996))" and also a co-inventor of the invention of a European Patent Application No. EP 0 596 524.
4. As described in the specification of the European Patent Application No. EP0596524, I obtained a novel protease which converts a hepatocyte growth factor (HGF) into an active type and a novel gene encoding thereof, for which a patent application (basic application) was filed in 1992. The above-mentioned protease is the same as "hepatocyte growth factor activator (HGFA)". Further, in Example 10 of the EP Application I disclosed antibodies against the HGFA and a method of producing thereof. The disclosed antibodies were obtained by the generally known standard method.

Then, I analyzed the obtained antibodies, and reported the analysis in the document "J. Biol. Chem., 271, No. 7, 3615-3618" in 1996. The monoclonal antibodies 7E10, P1-4, A-1, A-6, A-23, A-32, A-51 and A-75 were analyzed in the document, and all of them are the same as the antibodies obtained by the standard method as described in the EP Application No. EP0596524.

I assigned these monoclonal antibodies against the HGFA to Naka, et al. who are the inventors of the invention disclosed in the US Patent Application No. 10/000,096. Then, these antibodies were analyzed by Naka, et al, and it was confirmed as described in Example 4 of the aforementioned US Patent Application that they are the antibodies which recognize both the active and the inactive HGFA.

Now, I declare and confirm that the antibodies against HGFA described in the EP Application No. EP0 596 524 are the same as the antibodies described in J. Biol. Chem., 271, No. 7, 3615-3618, and that I assigned these antibodies to Naka, et al, who are the inventors of the US Patent application No. 10/000,096.

5. I declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful, false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States codes and that such willful, false statements may jeopardize the validity of the application or patent issuing therefrom.

Dated: August 23, 2004

By: Takeshi Shimomura
Takeshi Shimomura

Appendix

Publications of the declarant

1. A novel protease obtained from FBS-containing culture supernatant, that processes single chain form hepatocyte growth factor to two chain form in serum-free culture. (1992) Shimomura T., Ochiai M., Kondo J., and Morimoto Y., *Cytotechnology* 8: 219-229
2. Molecular Cloning and Sequence Analysis of the cDNA for a Human Serine Protease Responsible for Activation of Hepatocyte Growth Factor. (1993) Miyazawa K., Shimomura T., Kitamura A., Kondo J., Morimoto Y., and Kitamura N., *J. Biol. Chem.* 268: 10024-10028.
3. Activation of the Zymogen of Hepatocyte Growth Factor Activator by Thrombin. (1993) Shimomura T., Kondo J., Ochiai M., Naka D., Miyazawa K., Morimoto Y., and Kitamura N. *J. Biol. Chem.* 268: 22928-22932.
4. Heparin modulates the receptor-binding and mitogenic activity of hepatocyte growth factor on hepatocytes (1993) Naka D., Ishii T., Shimomura T., Hishida T., and Hara H., *Exp. Cell. Res.* 209, 317-323.
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6. Proteolytic activation of hepatocyte growth factor in response to tissue injury (1994) Miyazawa K., Shimomura T., Naka, D., and Kitamura N., *J. Biol. Chem.* 269, 8966-8970.
7. Activation of Hepatocyte Growth Factor by Blood Coagulation Factor XIIa and Comparison of Its Activity with That of Hepatocyte Growth Factor Activator. (1995) Shimomura, T., Miyazawa, K., Komiyama, Y., Hiraoka, H., Naka, D., Morimoto, Y., and Kitamura, N. *Eur. J. Biochem.* 229,257-261
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13. Functional characterization of kunitz domains in hepatocyte growth factor activator inhibitor type 2. (1998) Qin, L., Denda, K., Shimomura, T., Kawaguchi, T., and Kitamura, N., *FEBS Lett.* 436, 111-114
14. Distribution of Hepatocyte Growth Factor Activator Inhibitor Type 1 (HAI-1) in Human Tissues: Cellular Surface Localization of HAI-1 in Simple Columnar Epithelium and Its Modulated Expression in Injured and Regenerative Tissues. (1999) Kataoka, H., Suganuma, T., Shimomura, T., Itoh, H., Kitamura, N., Nabeshima, K., and Kono, M., *J. Histochem. Cytochem.* 47, 673-682
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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant : Naka, et al.
Appl. No. : 10/000,096
Filed : December 4, 2001
For : SPECIFIC ANTIBODY DIRECTED TO ACTIVE HEPATOCYTE
GROWTH FACTOR ACTIVATOR AND METHOD FOR USING THE
SAME

DECLARATION UNDER 37 C.F.R. § 1.132

Assistant Commissioner for Patents
Alexandria, VA 22313-1450

Dear Sir:

I, Daiji Naka, declare as follows.

1. I am a graduate of Tokyo University of Science, and I received my doctorate from the University.
2. I have been employed by Mitsubishi Chemical Corporation, Yokohama-shi, Kanagawa, Japan and its subsidiary, Zoegene Corporation, Yokohama-shi, Kanagawa, Japan as a researcher in the field of pharmaceutically application of hepatocyte growth factor and pharmaceutical development for genome medicine.
3. I am one of inventors of the above-identified application.
4. I have read and understood the patent application 10/000,096.
5. The following experiments were conducted by me or under my direct supervision and control.

EXPERIMENTS

With respect to the method of preparation of active hepatocyte growth factor activator (HGFA)-specific monoclonal antibody and analysis of reactivity thereof of the invention disclosed in the present application will be further explained in detail hereinbelow by referring to the contents of Examples 3 and 4 described in the specification of the present application.

(1) Preparation of active HGFA specific monoclonal antibody

A solution containing 100 µg of the 36 kDa HGFA or 100µg of the 98 kDa HGFA was subcutaneously and intraperitoneally administered to a Balb/c mouse with the same volume of complete Freund's adjuvant 6 times with 2-week intervals. The two kinds of active HGFA used here are those prepared according to the method described in Example 1 of the specification of the present application. After production of antibodies in the serum of the mouse was confirmed, a solution containing 100 µg of HGFA was administered into the caudal vein. Three days later, spleen was removed and spleen cells were fused with myeloma cells P3U1 by using polyethylene glycol 1500 according to "Monoclonal Antibody Experimental Manual " (Kodansha Scientific, 1987), introduced into wells of 96-well plate, added with HAT medium and cultured for 14 days. The cell fusion was performed four times (for 4 mouse), and hybridomas of a total of about 12000 clones were obtained.

Subsequently, hybridomas producing monoclonal antibodies specific to respective active HGFA in the medium were selected from the thus obtained hybridomas of 12000 clones.

First, culture supernatant of a hybridoma subjected to selection was added to an ELISA plate for screening active 36 kDa HGFA-type or active 98 kDa HGFA-type prepared in Example 2 described in the specification of the present application, and reactivity of the monoclonal antibodies existing in the culture supernatant was analyzed. In an amount of 100 µl/well of the culture supernatant of the hybridoma subjected to selection was added to the ELISA plate for screening each activity type and allowed to react at 4°C for 2 hours or longer.

Then, the plate was sufficiently washed with a PBS(-) solution containing 0.05% Tween 20 (hereafter, abbreviated as "PBST solution"), and then 100 µl of PBS(-) solution containing 1µg/ml HRP (horseradish peroxidase) -conjugated sheep anti-mouse IgG/Fc polyclonal antibody (DAKO) and 1% BSA was added to each well and allowed to react at room temperature for 1 hour. The plate was sufficiently washed with a PBST solution, and then a citrate-phosphate buffer (pH 5.0) containing 0.4 mg/ml orthophenylenediamine (OPD, Sigma, P-9029) and a 0.015-0.03% hydrogen peroxide solution was added and allowed to react at room temperature for color development. Then, the reaction mixture was added with a 1 NH₂SO₄ solution to stop the reaction and measurement was performed at a measurement wavelength of 490 nm and a reference wavelength of 650 nm.

Then, by using the culture supernatant of hybridoma producing monoclonal antibodies showing reactivity to the thus obtained active 36 kDa HGFA or active 98 kDa HGFA, the screening was performed in the same manner on the ELISA plate for screening inactive HGFA prepared in Example 2 of the specification of the present application to select a hybridoma that

does not show any reactivity to inactive HGFA (AHGA-A, AHGA-B, AHGA-C) were obtained.

Of the initially obtained hybridomas of 12000 clones, hybridomas which produce specific monoclonal antibodies that recognize active HGFA and do not recognize non-active HGFA were only these three (3) clones. That is, the probability of obtaining the active HGFA specific monoclonal antibody that do not recognize non-active HGFA of the invention of the present application was extremely low as 0.025% (3/12000). Of the other hybridomas, about 50% (6000/12000) were hybridomas which produce antibodies that recognize both the active and inactive HGFA. The remaining 50% hybridomas were either produce antibodies that does not recognize HGFA, or the hybridomas which do not produce antibodies at all.

Each obtained hybridoma was subjected to four times of cloning operations by a limited dilution method, and its culture supernatant was collected and subjected to affinity chromatography utilizing protein A (Amersham Pharmacia Biotec) to purify monoclonal antibodies.

The hybridoma clone AHGA-A was deposited on October 19, 2001 in the National Institute of Advanced Industrial Science and Technology, International Patent Organism Depository (Tsukuba Central 6, 1-1, Higashi 1-chome, Tsukuba-shi, Ibaraki-ken, 305-8566, Japan) under the deposition number of FERM BP-7779.

(2) Analysis of reaction specificity of active HGFA specific monoclonal antibodies comparing with existing monoclonal antibodies

Regarding active HGFA specific monoclonal antibodies derived from hybridoma clones AHGA-A, AHGA-B and AHGA-C which were prepared and purified in the above (1), their reactivities were analyzed.

For this purpose, 100 μ l of PBS(-) containing about 1 μ g/ml monoclonal antibodies derived from each of hybridoma clones AHGA-A, AHGA-B and AHGA-C and 1% BSA was added to each well of the ELISA plate for screening active 36 kDa HGFA or active 98 kDa HGFA, or ELISA plate for screening inactive HGFA, each of which was prepared in Example 2 described in the specification of this application, and allowed to react at 4°C for 2 hours or longer.

Subsequently, the plate was sufficiently washed with a PBST solution, and then 100 μ l of PBS(-) containing 1 μ g/ml HRP-conjugated sheep anti-mouse IgG polyclonal antibody (DAKO) and 1% BSA was added to each well and further allowed to react at room temperature for 1 hour. The plate was sufficiently washed with a PBST solution, and then a citrate-phosphate buffer (pH 5.0) containing 0.4 mg/ml orthophenylenediamine (OPD, Sigma, P-9029) and a 0.015-0.03% hydrogen peroxide solution was added and allowed to react at room temperature for color development.

Thereafter, a 1 N H₂SO₄ solution was added to the reaction mixture to stop the reaction and measurement was performed at a measurement wavelength of 490 nm and a reference wavelength of 650 nm. The monoclonal antibodies derived from hybridoma clones AHGA-A and AHGA-B were reactive to active 36 kDa HGFA, but not reactive to inactive HGFA. Further, those of the hybridoma clone AHGA-C were reactive to active 98 kDa HGFA, but not reactive to inactive HGFA. On the other hand, regarding the existing monoclonal antibodies (7E10, P1-4,

A-1, A-6, A-23, A-32, A-51, A-75) against HGFA, their reactivities to active 36kDa HGFA and inactive HGFA were analyzed. As a result, they all showed equivalent reactivities to both the active HGFA and inactive HGFA and did not have a reactivity specific to active HGFA unlike the monoclonal antibodies obtained in the present invention. Namely, it was found that the monoclonal antibodies which recognize specifically the active HGFA, can be obtained for the first time by the present invention.

It should be noted that the existing monoclonal antibodies (7E10, P1-4, A-1, A-6, A-23, A-32, A-51, A-75) against HGFA used here are the antibodies described in the J. Biol. Chem., 271, No. 7, 3615-3618. These antibodies are obtained by the inventors of the present invention from Shimomura, et al., who are the authors of the above-mentioned Reference, and a method of preparation of these antibodies is disclosed in the EP0 596 524 which was filed by Shimomura, et al.

(3) The active HGFA specific antibody of the present invention which is prepared by the method as described in detail above was obtained by a method different from that of the antibody described in the cited reference, and the antibody of the present invention can never be obtained without performing the screening with a definite and sure object to obtain an antibody which specifically recognize the active HGFA.

The present invention has been successful in obtaining the antibody which react specifically to the active HGFA by using the ELISA plate for screening active 36 kDa HGFA and active 98 kDa HGFA, or ELISA plate for screening inactive HGFA, each of which was prepared in Example 2 described in the specification of this application. Furthermore, the probability of success was an extremely low of about 0.025%. Generally, in the case of preparing a monoclonal antibody according to the standard method, the probability of obtaining the target monoclonal antibody is considered to be an average of about 50%, and, from this, it can be clearly understood that the probability of obtaining the antibody of the present invention is extremely low.

For example, in the case of preparing monoclonal antibodies according to the standard method, the cells, which were fused in about 24-well four (4) assay plates (a total of 96 wells), or about 96-well 10 assay plates (a total of 960 wells), are cultured (see, for example, "Monoclonal Antibodies – A practical Approach" (OXFORD UNIVERSITY PRESS), page 12, [Protocol 7], 5). In this case, even when it is assumed that hybridomas were obtained in all of the 9670 wells, and if the obtaining probability would be 0.025%, a calculation results in providing only 0.24 hybridomas which are the ones that can produce the target antibody, and this leads to the idea that substantially no target antibody can be obtained.

That is, the antibody according to the present invention is not the one that can be obtained by any one who obtains hybridoma by merely performing immunization according to the normal method, even if the antigen was known. Thus, it is considered that the antibody of the present invention can never be obtained without having a definite object to obtain the active HGFA specific monoclonal antibody by obtaining hybridomas in a great quantity of as many as 12000, constructing an analyzing system by the above-described specified ELISA method, and

performing the screening of the great quantity of hybridomas.

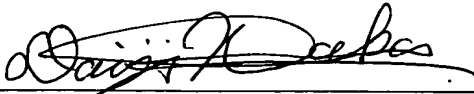
Further, the thus obtained antibody of the present invention provides the remarkable effect to be used for detection of a disease of a human, such as, glomerular nephritis, pancreatitis, cancer, myocardial infarction, angina pectoris, cerebral infarction, etc., as analyzed in Example 11 described in the specification of the present application. This effect can be obtained for the first time by virtue of the antibody of the present invention which reacts specifically to only the active HGFA.

On the contrary, regarding the known antibodies (7E10, P1-4, A-1, A-6, A-23, A-32, A-51, A-75) the preparation method thereof is described in Example 10 in the EP0596524 which describes that they were prepared by the standard method, and no special screening was performed. Furthermore, the character of each of these antibodies is analyzed in J. Biol. Chem., 271, No. 7, 3615-3618, but there is no description at all regarding analysis of a difference in reactivities to the active and the inactive HGFA. That is, from this fact it can be understood that at that time, there was no object to obtain the active HGFA specific antibody. In fact, these known antibodies were the antibodies all of which recognize both the active and the inactive HGFA as clarified in the above (2) by the inventors of the present invention.

As discussed above, the active HGFA specific antibody of the present invention was obtained by the particular method which is different from that of obtaining the known antibodies described in the cited reference, and it is not the one that could have been easily obtained by anyone by the known method.

6. I declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful, false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States codes and that such willful, false statements may jeopardize the validity of the application or patent issuing therefrom.

Dated: August, 23/2004

By: 
Daiji Naka

Appendix 1

Publications of the declarant

1. Isolation and conformational analysis of fragment peptide corresponding to the heparin-binding site of hepatocyte growth factor. Aoyama-H; Naka-D; Yoshiyama-Y; Ishii-T; Kondo-J; Mitsuka-M; Hayase-T *Biochemistry*. 36(33): 10286-91, 1997
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Monoclonal Antibodies

A Practical Approach

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Protocol 6 continued

4. Centrifuge cells for 5 min at 400 g, wash twice in serum-free DMEM, and resuspend in 10 ml of the same medium.
5. Count viable lymphoid cells in haemocytometer. Spleens from immune mice yield about 10^8 cells, from rats $3-5 \times 10^8$ cells, and the mesenteric nodes of rats, up to 2×10^8 cells.

8 Cell fusion

A number of protocols have been described for the generation of mouse hybridomas and workers have their own particular protocol. We have used a standard procedure for the fusion of mouse and rat myelomas with considerable success over the last twenty years and this is described in *Protocol 7*. Basically, 10^8 lymphocytes are mixed with 2×10^7 mouse myeloma cells or 5×10^7 Y3 cells in a round-bottomed tube and pelleted by centrifugation. Then the cells are fused by the addition of 1 ml of 50% PEG 1500 and plated into HAT selection medium to allow the growth of the hybridomas generated.

Protocol 7**Hybridoma formation****Equipment and reagents**

- Sterile capped 10 ml tube
- 24-well or 96-well plates (Nunc)
- Dulbecco's modified Eagle's medium (DMEM)
- Fetal calf serum (FCS): inactivated by heating for 45 min at 56°C and tested for ability to support the growth of hybridomas
- PEG solution: weigh 50 g of polyethylene glycol (1500 molecular weight) into a capped 200 ml bottle, add 1 ml of water, and autoclave for 30 min at 120°C . Cool to about 70°C , then add 50 ml of DMEM, mix, and, after cooling to ambient temperature, adjust the pH to about 7.2 with NaOH (mixture should be coloured orange). Store as 1 ml aliquots at -20°C .
- HAT selection medium: prepare $100 \times$ HT by dissolving 136 mg hypoxanthine and 38.75 mg thymine in 100 ml of 0.02 M NaOH pre-warmed to 60°C . Cool, filter sterilize, and store at -20°C in 2 ml aliquots. Prepare $100 \times$ A by dissolving 1.9 mg aminopterin in 100 ml of 0.01 M NaOH, then filter sterilize, and store in 2 ml aliquots at -20°C . Prepare HAT medium by adding 2 ml of HT and 2 ml of A to 200 ml of DMEM containing 20% FCS.
- HT medium: add 1 ml of HT to 100 ml of DMEM containing 10% FCS
- Feeder cells for fusion cultures and cloning of hybridomas (essential for fusions using rat myelomas): rat fibroblast cell lines derived from the xiphisternae of various strains are suspended in DMEM and irradiated with about 30 Gy (3000 rad) of X- or gamma rays, then frozen in 95% FCS, 5% DMSO, and stored in liquid nitrogen as aliquots of 5×10^6 cells. Use one aliquot for each 200 ml of HAT or HT medium. Alternatively, use thymocytes from spleen donors.
- Freezer medium: freshly prepared 5% dimethyl sulfoxide, 95% FCS

Protocol 7—continued

Method

1. Mix 10^8 viable lymphocytes with 2×10^7 mouse myeloma cells or 5×10^7 rat myeloma cells in a 10 ml capped centrifuge tube and centrifuge for 3 min at 400 g.
2. Pour off the supernatant, drain carefully with a Pasteur pipette, then release the cell pellet by gently tapping the tube on the bench.
3. Stir 1 ml of PEG solution, pre-warmed to 37°C , into the pellet over a period of 1 min. Continue mixing for a further minute by gently rocking the tube.
4. Dilute the fusion mixture with DMEM (2 ml over a period of 2 min and then 5 ml over 1 min).
5. Centrifuge for 3 min at 400 g, then resuspend the cells in 200 ml of HAT selection medium (containing feeder cells where necessary), and plate 2 ml aliquots into four 24-well plates or 200 μl aliquots into ten 96-well plates. Incubate at 37°C in 5% CO_2 .
6. Examine plates 7–14 days later for the presence of hybridomas and screen for the presence of specific antibodies (see *Protocols 10–12*).

9 Screening hybridoma culture supernatants for specific antibody

The fusion cultures are examined for the growth of hybridoma colonies from seven days onwards. Mouse hybridomas tend to grow initially as discrete colonies (sphaeroids) whereas hybridomas produced with the Y3 myeloma are quite diffuse and not easy to recognize. With further growth the Y3 hybridomas form discrete colonies that can be quite firmly attached to the plastic. Screening is normally carried out initially at 10–14 days post-fusion by removing samples of about 200 μl of supernatant:

The importance of the initial screening assay cannot be overemphasized because it is on the basis of the result that the hybridoma colony is picked, expanded, and cloned. Many different procedures have been used to screen hybridoma culture supernatants from direct binding to cells or tissues using fluorescent, radiolabelled (see *Protocol 9*), enzyme-linked, or otherwise tagged second antibodies (anti-mouse, anti-rat, anti-sheep, etc.) to detect bound monoclonal antibody, to functional assays, e.g. inhibition of binding of ligand to its receptor. The important feature of the assay(s) used is that it is quick, reliable, and specific. Most hybridomas grow rapidly with a generation time of 10–12 hours (rat) to 15–24 hours (mouse) so that long-term assays are precluded. Assays that determine binding to antigen, whether present on/in cells, to proteins or peptides coated onto plastic multiwell plates or pins or to tissue sections are to be preferred to biological assays that cannot be performed in one day.

9.1 Antigen coated multiwell plates

Tests that can be carried out on 96-well plates coated with antigen either as protein or as cell monolayers are ideal for these initial screens. Bound anti-

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